Inhibition of corn root membrane ATPase activities by oryzalin

Shu-I Tu*, Deidre Patterson, David Brauer and An-Fei Hsu

Abstract The effects of oryzalin, a herbicide, on the H^+-ATPase activities of microsomal membranes from corn roots were investigated. The presence of the herbicide at micromolar concentrations did not significantly affect the hydrolytic activity of both tonoplast and plasma membrane H^+-ATPases. However, the coupled proton pumping of the H^+-ATPases in crude microsomes could be inhibited by the chemical. Similarly, the presence of micromolar levels of oryzalin also caused a steady decrease in ATP-driven proton pumping in purified tonoplast and plasma membrane vesicles. Kinetic analysis indicated that oryzalin decreased the initial proton pumping rate without significant effects on the proton leakage of both energized and de-energized membranes. In contrast, the presence of sub-optimal concentrations of CCCP, a conventional protonophore, inhibited the apparent proton pumping by increasing the proton leakage of the membrane. The results indicate that oryzalin may upset the generation and the utilization of ATP in corn root cells. The observed preferential inhibition on the proton pumping is consistent with a previously proposed indirect coupling mechanism for the root membrane H^+-ATPases.

Key words Herbicidal action, H^+-ATPase, plasma membrane, proton transport, tonoplast vesicles, *Zea mays*.

Abbreviations AO, acridine orange; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DMSO, dimethyl sulfoxide; k_1, rate constant for measuring proton pumping retardation of energized (with ATP hydrolysis occuring) membranes; k_2, rate constant for measuring proton leakage of de-energized (with ATP hydrolysis silent) membranes; R_{ATP}, initial rate of ATP hydrolysis; R_0, initial proton pumping rate; t, time; δ, absorbance change; δ_s, absorbance change at steady state. (Reference to brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned).

INTRODUCTION

Oryzalin, 4-(dipropylamino)-3,5-dinitrobenzene sulfonamide, is used primarily as a pre-emergence herbicide (Parka and Soper, 1977). The toxicity of oryzalin is thought to originate primarily from its ability to disrupt microtubules (Bartels and Hilton, 1973; Fedtke, 1982) and therefore cell division of both monocotyledonous and dicotyledonous plants (Cleary and Hardham, 1988). The faster uptake of oryzalin by monocotyledonous than dicotyledonous species may be related to the apparent preference of the chemical against the former (Upadhyaya and Nooden, 1987). This herbicide also inhibits the respiration of excised roots and calcium uptake by isolated plant mitochondria (Parka and Soper, 1977; Corbett *et al.*, 1984). Thus, the compound also appears to be an inhibitor for the generation of ATP by oxidative phosphorylation. However, it remains to be established whether oryzalin can affect the coupled functions of membrane-bound H^+-ATPases which are important in providing the energy to support the transport of nutrients and metabolites in root cells.

The H^+-ATPases of the plasma membrane and tonoplast membrane of root cells catalyze the

hydrolysis of Mg-ATP and utilize released chemical free energy to support the vectorial movement of protons across the membranes (Sze, 1984). The resulting transmembranous proton electrochemical potentials, $\Delta\mu_{H+}$, may serve as the driving force for nutrient uptake from the soil environment across the plasma membrane and the redistribution of nutrients and metabolites between the cytoplasm and vacuole across the tonoplast membrane (Sze, 1984). Plasma membrane H^+-ATPase is known to contain only one type of 100 kDa subunit (Briskin, 1990). In the catalytic pathway of ATP hydrolysis, the enzyme forms a phosphorylated intermediate which may be linked to the activation of proton pumping. Freshly isolated corn root plasma membrane vesicles rapidly lose their proton pumping but not ATP hydrolysis activity associated with the vanadate-sensitive H^+-ATPase probably due to the action of proteases (Gallagher and Leonard, 1987) and/or phospholipases (Brauer *et al.*, 1988). This pumping activity can be restored through reconstitution with phospholipid liposomes (Brauer *et al.*, 1988). The stoichiometry of H^+/ATP of plant plasma membrane H^+-ATPase has been determined under certain conditions (Brauer *et al.*, 1989; Briskin and Reynolds-Niesman, 1991). However, the exact mechanism governing the conversion of ATP hydrolysis energy into $\Delta\mu_{H+}$ remains to be determined (Briskin and Hanson, 1992).

Tonoplast H^+-ATPase, on the other hand, does not form a phosphorylated intermediate in its reaction pathway (Sze, 1984; Nelson and Taiz, 1989). The enzyme contains many different subunits and is anion-sensitive. While the electrogenic proton pumping associated with the enzyme can be easily detected in isolated tonoplast vesicles, its reconstitution with phospholipid vesicles was only recently accomplished (Ward *et al.*, 1992). It was reported in a series of studies from this laboratory that the ATP hydrolysis and its coupled H^+-pumping exhibited differential sensitivity toward temperature change (Tu *et al.*, 1988), nitrate anions (Tu *et al.*, 1987), mercuric cations (Tu *et al.*, 1988), and fluorescamine treatment (Tu *et al.*, 1990). These observations were used to support an indirect coupling mechanism for the enzymatic activities (Tu *et al.*, 1990, 1992).

In this work, we investigated the effects of oryzalin on the activities of membrane-bound ATPases from corn roots. We found that the presence of micromolar levels of oryzalin did not significantly affect the ATP hydrolysis activity of neither plasma membrane ATPase nor tonoplast membrane ATPased. However, the coupled proton pumping activities of both ATPases were significantly inhibited. Kinetic analysis revealed that the mode of action of oryzalin is quite different from that of CCCP, a common protonophore. These results suggest that the interactions with membrane-bound ATPases may constitute an alternative mechanism of action for the herbicidal activity of oryzalin.

RESULTS

Effects of oryzalin on microsomal ATPase activities

As described in our earlier reports (Tu *et al.*, 1987; Brauer *et al.*, 1988), the microsomal fractions obtained from corn roots contain minimal activities of molybdate-sensitive phosphatase, oligomycin-sensitive ATPase, and deoxycholate-stimulated UDPase. The membrane contained both vanadate-sensitive and nitrate-sensitive ATP hydrolyzing activities. The presence of 50 mM KNO_3 and 0.2 mM vanadate, inhibited 95% of the total ATP hydrolysis (102 nmol P_i min^{-1} mg^{-1}) found in 50 mM KCl at pH 6.5. Thus, the ATP hydrolyzing activity (at pH 6.5) of the microsomal vesicles was primarily derived from plasma membrane and tonoplast membrane ATPases. A total inhibition of the ATP-supported proton pumping (0.022 ΔA min^{-1} mg^{-1}) by the microsomal fraction in 50 mM KCl required the presence of both 0.2 mM vanadate and 2 mM KNO_3. Thus, only the plasma membrane H^+-ATPase (vanadate-sensitive) and the tonoplast H^+-ATPase (nitrate-sensitive) were involved in the observed H^+ pumping activity.

Our preliminary experiments also showed that the presence of oryzalin, up to 10 μM, had no significant effect on ATP hydrolysis catalyzed by the corn root microsomal fraction in solutions containing 50 mM KCl, 50 mM KCl with 0.2 mM vanadate, or 50 mM KNO_3. However, the total, nitrate-sensitive, and vanadate-sensitive proton pumping activities, as measured by the initial rates, were inhibited. With 10 μM oryzalin, a decrease of approximately 60% of the total, the nitrate-sensitive, and vanadate-sensitive proton pumping activities were noted. These results would suggest a similar sensitivity of both proton pumping systems to the presence of oryzalin. In order to further quantify its inhibition, the effects of oryzalin on ATP-supported proton pumping in isolated subcellular membrane vesicles were investigated.

Plasma membrane vesicles obtained by the described procedure, exhibited ATP-driven proton pumping activity. The presence of oryzalin, up to 5 μM, slightly enhanced ATP hydrolysis by about 10% (*fig.* 1 A). The initial rate of proton pumping, on the other hand, was inhibited by 25% at 5 μM oryzalin. These results, at first glance, are consistent with the notion that oryzalin may increase the proton leakage of the membrane and thus, stimulate the ATP hydrolysis (uncoupling effect). The kinetic model of describing the proton movement provided quantitative estimation of the membrane proton leakage from the values calculated for rate constants, k_1 and k_2. As shown in figure 1 B, oryzalin did not significantly change these rate constants indicating a minimal effect on the proton leakage of the membrane vesicles.

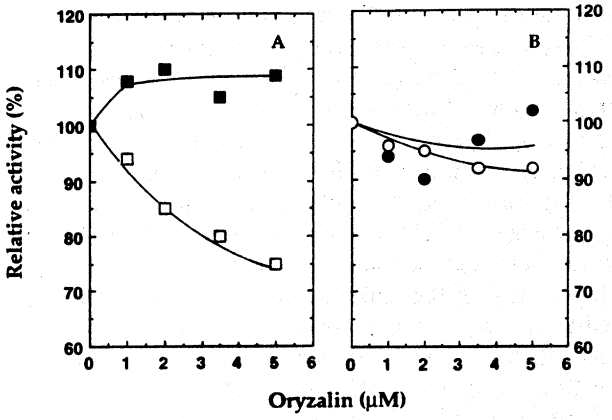

Figure 1. *Effects of oryzalin on coupled activities of the plasma membrane H^+-ATPase.* The plasma membrane vesicles obtained by sucrose density centrifugation were incubated with oryzalin at indicated concentrations for 10 min in the assay media before the addition of ATP. **A**, the initial rates of ATP hydrolysis (R_{ATP}, filled squares) and proton pumping (R_0, open squares) were determined as described. The relative activities were calculated by assigning the values obtained without oryzalin as 100%. Typical 100% values of R_{ATP} and R_0 were 220 nmol P_i released min^{-1} mg^{-1} and 0.065 ΔA at 492.5 nm min^{-1} mg^{-1}, respectively; **B**, the relative proton leak rate constants obtained during the build-up of the gradient and after ATP hydrolysis quenched by hexokinase are shown as filled circles and open circles, repectively. The k_1 (0.282 min^{-1}) and k_2 (0.557 min^{-1}) obtained in the absence of oryzalin were assigned as 100%.

Effects of oryzalin on tonoplast ATPase activities

The tonoplast vesicles isolated by equilibrium sucrose density gradient centrifugation, as previously described (Tu *et al.*, 1987), were essentially free from non-tonoplast activities including plasma membrane, Golgi apparatus, ER, mitochondria, and molybdate-sensitive phosphatase. The effects of oryzalin (0 to 25 μM) on the initial rates of coupled activities of the tonoplast H^+-ATPase are shown in figure 2 A. With oryzalin up to 5 μM, the initial ATP hydrolysis rate (R_{ATP}) was only slightly affected. However, the initial proton pumping rate (R_0) showed a 40% decrease at 5 μM oryzalin. The addition of 25 μM of oryzalin, completely inhibited the proton pumping, yet about 80% of ATP hydrolysis still remained. The decrease in the initial proton pumping rate, R_0, as calculated by the steady-state kinetics, was primarily derived from a decrease in δ_s. The rate constant k_1 was not significantly affected by oryzalin suggesting that proton leakage of the energized membrane was not significantly altered by oryzalin (*fig.* 2 B). Because the rate of ATP hydrolysis was not affected, the decrease in the initial pumping rate would suggest a decrease in the coupling, expressed as the stoichiometric constant "m", between ATP hydrolysis and proton pumping.

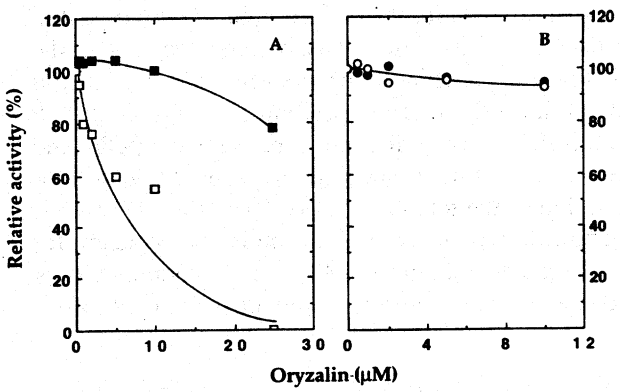

Figure 2. *Effects of oryzalin on coupled tonoplast H^+-ATPase activities.* The tonoplast vesicles obtained by the equilibrium sucrose density centrifugation were incubated with oryzalin of indicated concentrations in the assay media for 10 min before the addition of ATP. **A**, the initial rates of ATP hydrolysis (solid squares) and proton pumping (open squares) were determined as described. The relative activities were calculated by assigning the values with zero oryzalin addition as 100%. Typical 100% values of R_{ATP} and R_0 were 320 nmol P_i released min^{-1} mg^{-1} and 2.02 ΔA at 492.5 nm min^{-1} mg^{-1}, respectively. **B**, the relative proton leak rate constants obtained during the build-up of the gradient and after ATP hydrolysis quenched by hexokinase are shown as filled circles (k_1) and open circles (k_2), respectively. The k_1 (0.508 min^{-1}) and k_2 (1.423 min^{-1}) obtained in the absence of oryzalin were assigned as 100%.

The proton leakage of the de-energized membrane, k_2, as determined by the rapid quenching of the

hydrolysis activity with hexokinase and glucose, also was not significantly affected by the presence of oryzalin (*fig.* 2 B). Thus, oryzalin appears not to affect the integrity of the tonoplast membrane under both energized and de-energized conditions, a situation similar to that observed in plasma membrane vesicles.

Since tonoplast H^+-ATPase is known to contain multiple subunits, the observed decrease of proton pumping activity could be the result of an oryzalin-induced solubilization/dissociation of the subunit containing the active site for ATP hydrolysis. The relative insensitivity of the ATP hydrolysis to oryzalin (*fig.* 2 A) would imply that the solubilization should not change the catalytic activity of the subunit. To test this possibility, experiments shown in table 1 were conducted. The tonoplast vesicles were allowed to incubated in the proton pumping medium for 10 min with or without 25 μM oryzalin at room temperature. The suspension was then centrifuged at 84,000 $\times$ *g* for 1 h at 0°C. The presence of 25 μM oryzalin inhibited about 20% of the ATP hydrolyzing activity and almost abolished all the proton pumping activity (*fig.* 2 A). If the dissociation were the cause for the observation, then for the vesicles treated with oryzalin, all the ATP hydrolyzing activity would remain in the supernatant. As shown, the presence of oryzalin did not significantly alter the percentage of the total ATP hydrolyzing activity distributed between the pellet and the supernatant fractions. Thus, oryzalin-induced solubilization of the tonoplast H^+-ATPase is unlikely. This solubilization related complication is not a concern for the plasma membrane H^+-ATPase that contains only one type of transmembrane subunit (Briskin, 1990).

Comparison with the effects of protonophore

The kinetic analyses indicated that oryzalin affects the proton pumping primarily by changing the coupling with ATP hydrolysis (the value of "m") but not the membrane leakage (k_1 and k_2). This result is quite different from that expected from a conventional protonophore/uncoupler which can rapidly shuttle protons across membranes, or increase membrane proton conductance/leakage. It is predicted then that CCCP, a protonophore, should increase the values of k_1 and k_2 associated with the proton movement. We have shown in a previous study of tonoplast H^+-ATPase (Tu *et al.*, 1987) that the presence of optimal concentrations of CCCP does abolish the steady-state proton gradient and substantially enhances ATP hydrolysis rate, indicating an increase in the membrane proton leakage.

To assure that oryzalin indeed affects the proton transport differently from that of protonophores, we investigated the kinetic consequences of adding sub-optimal concentrations of CCCP to the proton pumping process of the membrane. Visual comparison of the time courses of proton pumping recorded by the spectrometer, yielded no easily detectable difference between the effects of oryzalin and CCCP. The chemicals all appeared to decrease the steady-state level of proton transport (δ_s). However, analysis of the build-up time courses of proton gradient indicated that the presence of sub-optimal concentrations of CCCP indeed increased the value of k_1 (*tab.* 2). A substantial enhancement of the leak rate constant of de-energized membrane (k_2) by the presence of CCCP was also noted. It should be mentioned that the ATP hydrolysis rate (R_{ATP}) was not significantly affected by the presence of the low levels of CCCP described in table 2.

Table 1. *Effects of oryzalin on the distribution of ATP hydrolyzing activity in the tonoplast suspension.* The incubation of the tonoplast vesicles and the measurement of ATP hydrolysis rate were performed as described in figure 2 A. In agreement with the results shown in figure 2 A, 25 μM oryzalin inhibited about 20 % of the ATP hydrolysis and nearly all the proton pumping (row of total). In an identical but separated set of samples, the vesicles were incubated with indicated concentrations of oryzalin (no ATP addition) and then centrifuged. After centrifugation, the ATP hydrolysis activity of the pellet and supernatant fractions were determined. The relative recovery of the activity in centrifuged fractions were shown in parentheses. The 1 h centrifugation at 0°C did not change the ATP hydrolyzing activity of the vesicles as judging from the total recovery of the ATP hydrolyzing activity. The data shown represented the average of triplicated experiments.

	ATP hydrolyzing activity (*nmol P_i min^{-1} mg^{-1}*)	
	+ 0 μM oryzalin	+ 25 μM oryzalin
Before centrifugation		
total	404 ± 20 (100.0%)	333 ± 17 (100.0%)
After centrifugation		
recovered in pellet	314 ± 17 (77.7%)	257 ± 12 (77.2%)
recovered in supernatant	87 ± 4 (21.5%)	81 ± 4 (24.3%)
total recovery	401 ± 25 (99.2%)	338 ± 22 (101.5%)

Table 2. *Effects of CCCP on tonoplast membrane proton leakage.* The proton pumping kinetics were determined as described in text under the conditions of figure 4. Tonoplast membrane vesicles were allowed to incubate first with CCCP of indicated concentrations for 10 min before the addition of ATP. The relative values of rate constants were determined by assigning these obtained without additions (k_1 = 0.508 min^{-1}, k_2 = 1.423 min^{-1}) as 100%. The averages of three independent runs were listed.

CCCP (μM)	Energized membrane, k_1 (*relative, ± 2%*)	De-energized membrane, k_2 (*relative, ± 2%*)
0.0	100	100
0.04	107	114
0.08	116	215

Effects of oryzalin on enzyme kinetics

A comparison between figures 1 A and 2 A indicated that the proton pumping associated with the tonoplast H^+-ATPase, appears to be more sensitive to oryzalin. Thus, further experiments were performed to characterize the effects of oryzalin on the tonoplast vesicles. As described in previous reports (Tu *et al.*, 1988; 1989), both the hydrolysis and pumping activities of the tonoplast H^+-ATPase may be satisfactorily described by a simple Michaelis-Menten kinetics. The K_ms of the two activities, measured at pH 6.5 and 22°C by ATP concentration dependence, are 0.1 and 0.2 mM for ATP hydrolysis and proton pumping, respectively. As expected, the insensitivity of ATP hydrolysis to oryzalin (up to 10 μM) resulted

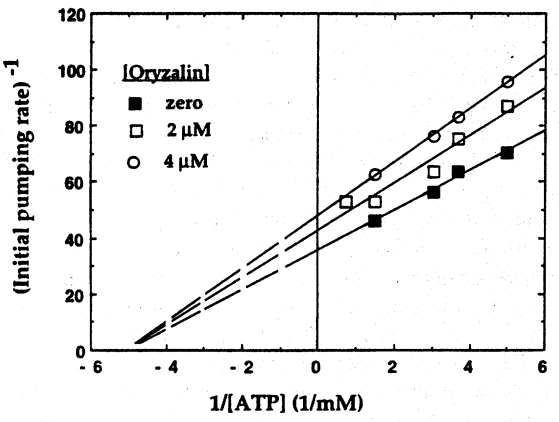

Figure 3. *Enzyme kinetic effects of oryzalin on H^+ pumping.* Different concentrations of ATP were used to initiate the H^+ pumping of the tonoplast vesicles treated with various amounts of oryzalin by the procedures described in figures 2 and 4. Double reciprocal plots of two independent runs (errors less than 5%) were then constructed and fitted with a simple Michaelis-Menten kinetic scheme. The linearity of the lines was better than 99 ± 1%.

Table 3. *Effects of oryzalin on enzymatic parameters of H^+-pumping in tonoplast vesicles from corn roots.* The initial proton pumping rates (R_0) induced by changing ATP concentration (0.1 to 1.3 mM) of the tonoplast H^+-ATPase were measured as described in text except the vesicles were allowed to incubate with oryzalin for 10 min before the addition of ATP. The numerical values listed represent an average of three independent runs with an error range as ± 5%.

Oryzalin (μM)	K_m (mM)	V_{max} ($\Delta A\ min^{-1}\ mg^{-1}$)
0	0.200	0.281
2	0.199	0.234
4	0.199	0.207

in no change of either K_m or V_{max} of the process (data not shown). The presence of oryzalin also did not change the Michaelis-Menten dependence of the proton pumping (*fig.* 3). The inhibition appeared to assume a noncompetitive mode, *i.e.*, showing a decrease in V_{max} but not in K_m of the proton pumping process (*tab.* 3). From the change in V_{max}, a K_i value around 10 μM was calculated. The results suggest that the presence of oryzalin may not have a significant effect on the binding and the hydrolysis of ATP but appear to change the catalytic turnover process(es) leading to proton pumping by the tonoplast H^+-ATPase.

DISCUSSION

The results of the present study indicate that oryzalin at micromolar concentrations can affect the coupled functions of membrane H^+-ATPases from corn roots. Specifically, it did not significantly inhibit the ATP hydrolysis catalyzed by tonoplast and plasma membrane H^+-ATPases. However, the proton pumping activity of the H^+-ATPases was inhibited by this herbicide. The steady-state kinetic analysis suggested that oryzalin achieved its inhibition by decreasing the coupling, expressed as "m" in equation (*4*), between ATP hydrolysis and proton pumping. By the same analysis, CCCP, a conventional protonophore, was shown to slow down the apparent proton pumping mainly by increasing membrane proton leakage. Although these two chemicals had a similar effect on the time course of proton pumping, the detail kinetic analyses indicate a very different origin of the effects. It should be mentioned that the disruption of the proton gradients was previously observed in oat systems (Ratterman and Nelson, 1987). However, no

detailed investigation on the origin of the observation was offered in their study.

Proton pumping may be coupled to ATP hydrolysis either directly or indirectly. For a direct coupling mechanism, the molecular pathway leading to proton pumping must share at least one common step with the pathway of ATP hydrolysis (Mitchell, 1975; 1985). Consequently, perturbation to one process should induce an identical response from the other coupled event. For an indirect coupling which may be achieved through conformational interactions (Boyer, 1988), two coupled events can exhibit different responses to external perturbations (Briskin and Hanson, 1992; Tu *et al.*, 1992). The results that proton pumping is preferentially inhibited by oryzalin suggest that the coupling between proton pumping and ATP hydrolysis in corn root H^+-ATPases may be indirect.

It has been reported that the presence of 5 to 10 μM oryzalin completely abolished the appearence of microtubules in suspension-culture plant cells (Laporte *et al.*, 1993) and mitochondrial Ca^{2+} uptake (Corbett *et al.*, 1984). This dosage is similar to the effective concentrations of oryzalin on ATPase activities reported in this study. Considering the importance of ATPase activities in cell functions, the observed inhibitions may be included as possible alternatives for the herbicidal activity of oryzalin.

METHODS

Isolation of crude microsomes. Unless indicated otherwise, the isolation of membranes described in this study was performed between 0 and 4 °C. The growth of corn seedlings and the isolation of microsomes were described in our previous work (Brauer *et al.*, 1988). Briefly, approximately 60 g of freshly excised 3-day old corn roots (*Zea mays* hybrid FRB-73 or WF7551, Illinois Foundation Seeds or Custom Farm Seed) were homogenized with a chilled mortar and pestle in a volume of 180 ml of ice-cold homogenization medium which contained 0.3 M sucrose, 5 mM EDTA, 5 mM DTT, 5 mM 2-mercaptoethanol and 0.1 M Hepes, pH 7.5. The homogenate was filtered through 4 layers of cheesecloth and the brei collected. For microsomal preparation, the filtered homogenate was first centrifuged at 6,000 × *g* for 15 min. The supernatant was centrifuged at 90,000 × *g* for 40 min and the pellet was washed once by the fresh homogenizing medium and collected by another centrifugation at 90,000 × *g* for 40 min. The final pellet after suspended to about 2 mg protein ml^{-1} in the same homogenizing medium was used as the microsomes.

Separation of plasma membrane and tonoplast vesicles. The isolation of the plasma membrane vesicles followed the procedure described by Brauer *et al.* (1992). An aliquot of 4 ml of the microsomal suspension was layered over a discontinuous density gradient consisting of 10 ml each of 34 and 42% (w/w) sucrose buffered with 5 mM Hepes, pH 7.5. After being centrifuged at 100,000 × *g* for 150 min, the membranes collecting at the interface between 34 and 42% sucrose steps were removed, diluted 3- to 4-fold with 5 mM Hepes (pH 7.5) and then centrifuged at 100,000 × *g* for 90 min. The final pellet was resuspended in 20 mM Hepes (pH 7.5), 0.25 M sucrose, and 10% (w/v) glycerol at 5 mg protein ml^{-1}.

For tonoplast vesicle preparation, the microsomal suspension was overlaid on a 15 to 45% (w/w) linear sucrose gradient containing 1 mM DTT and 5 mM Hepes, pH 7.5 as previouly described (Tu *et al.*, 1987). After centrifuged at 84,000 × *g* for 16 to 18 h at 4°C, the fractions with nitrate-sensitive H^+-ATPase activities obtained between 17-22% sucrose were pooled and used as tonoplast vesicles.

Measurement of membrane ATPase activities. The ATP hydrolysis catalyzed by microsomal, plasma membrane, and tonoplast vesicles was determined by the addition of 2.0 to 2.5 mM ATP to the membrane vesicles in a solution containing 50 mM KCl (tonoplast ATPase) or 50 mM KNO_3 (plasma membrane ATPase), 5 mM glucose, 2.5 mM $MgSO_4$, 1 mM EGTA, 10 μM AO, 17.5 mM Mes (pH 6.5), 0.1 mM ammonium molybdate and other chemicals as specified. After an incubation at room temperature for 10 min, the reaction was initiated by the addition of ATP. The reaction was terminated at the end of 10 min by the addition of ice-cold 5% TCA. The release of inorganic phosphate determined by Malachite-green molybdate complexation method (Tu *et al.*, 1987) was used to measure ATP hydrolysis. We previously reported that the ATP hydrolysis by purified microsomal subcellular membrane vesicles maintained a constant rate during the build-up and after attaining a steady-state of proton gradient (Tu *et al.*, 1987; Brauer *et al.*, 1989, 1992). Thus, the average hydrolysis rate over a period of 10 min was used to represent the initial rate of ATP hydrolysis (R_{ATP}).

The coupled proton movement was followed from the absorbance change of AO at 492.5 nm after the addition of ATP. The digitized time course (sampling time = 1 s) recorded by a Beckman DU-70 spectrometer (response time = 0.05 s) was directly transferred to an IBM PC for processing to determine kinetic parameters defined by a steady-state mechanism (Tu *et al.*, 1987). The net proton transport at time t after ATP addition can be represented by:

$$d\delta/dt = R_0 - k_1 \delta \qquad (1)$$

where δ, R_0, and k_1 represent the extent of proton transport, the initial proton pumping rate, and pumping inhibition

constant, respectively. The inhibition may include proton leakage of energized membrane (in the presence of ATP hydrolysis), back pressure, and pump slippage. The fact that ATP hydrolysis assumed a constant rate during the build-up of proton gradient indicated a lack of a back pressure effect in present system. Thus, k_1 may be considered mainly as a measure of proton leakage associated with the "energized membrane". At steady-state, the net proton transport rate approaches zero. Therefore,

$$R_0 = k_1 \delta_s \qquad (2)$$

in which δ_s represents the extent of proton transported at steady-state. The following is obtained after substituting (2) into (1) and integrating:

$$\ln(1 - \delta/\delta_s) = k_1 t \qquad (3)$$

Since both δ and δ_s are easily measured, the initial proton pumping rate R_0 can be determined without any subjective error often encountered by drawing a tangent through the initial portion of the pumping time course. Because proton pumping is supported by ATP hydrolysis, the initial rates of the two processes can be related as:

$$R_{ATP} = m R_0 \qquad (4)$$

in which "m" is the stoichiometric ratio or extent of coupling between proton pumping and ATP hydrolysis. A rapid quenching of ATP hydrolysis can discharge the proton gradient δ_s by leakage through de-energized membrane. Hexokinase induced phosphorylation of glucose may be utilized to rapidly exhaust added ATP. As demonstrated in our previous work (Tu *et al.*, 1987), the leak back of protons follows a simple first order decay:

$$d\delta/dt = -k_2 \delta \quad \text{or} \quad \ln(\delta/\delta_s) = -k_2 t \qquad (5)$$

Here, δ represents the residual gradient at time t after the quenching. It should be noted k_2 represents the proton leakage constant of de-energized (ATP hydrolysissilent) membrane. An illustration of applying the described procedure to characterize the H^+ movement is shown in figure 4. In practice, constants k_1 and k_2 were determined from digitized time courses directly. Because the structural features of the membrane need not be the same under different energetic conditions, it is not suprising that the k_1 and k_2 may assume different values.

Oryzalin and CCCP treatments. To test the effects of oryzalin and CCCP on the ATPase activities, the membrane vesicles were allowed to incubate with the chemicals at specified concentrations for 10 min in the assay solution

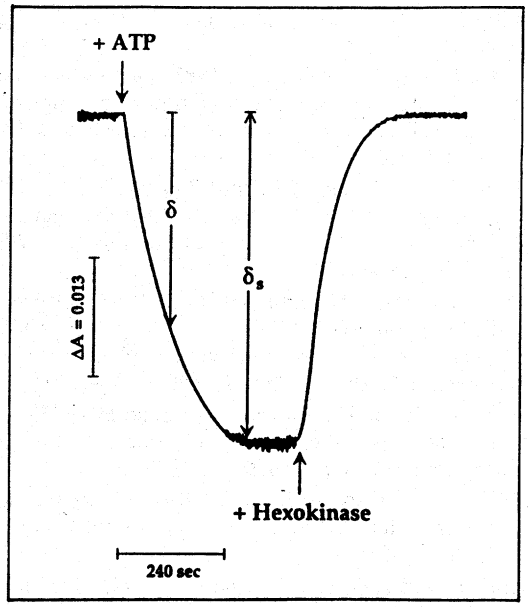

Figure 4. *Steady-state treatment of ATP dependent H^+ movement.* The proton pumping of corn root tonoplast vesicles containing 540 μg of protein was initiated by the addition of 20 μl of 0.2 M ATP. After reaching the steady-state (δ_s), 100 units of hexokinase was added to quench the ATPase activity. As shown in the figure, δ represents either the generated or remained proton gradients during the growth and decay phases of H^+ movement, respectively. One unit of hexokinase activity was defined as the ability to phosphorylate 1 μmol of glucose per min under employed conditions.

at room temperature before the addition of ATP. Since the chemicals were dissolved in either ethanol or DMSO, the same volumes of the organic solvents were also included in the control experiments. The volume of these solvents in the assay media was less than 0.5% of the total volume and showed no apparent effect on measured ATPase activities.

Other methods. The protein concentration was determined according to a modified Lowry method using bovine serum albumin as the standard (Bensadoun and Weinstein, 1976). Oryzalin, was obtained from Chem Service (Chester, PA).

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